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Title The MK2 cascade regulates mGluR-dependent synaptic plasticity and reversal learning

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Highlights

- The MK2 cascade regulates mGluR-mediated synaptic plasticity
- The mGluR-p38-MK2 cascade as a novel mechanism to prime LTP
- MK2 knockout mice are unable to reverse a previously learned hippocampal-dependent spatial task

Abstract

The ability to either erase or update the memories of a previously learned spatial task is an essential process that is required to modify behaviour in a changing environment. Current evidence suggests that the neural representation of such cognitive flexibility involves the balancing of synaptic potentiation (acquisition of memories) with synaptic depression (modulation and updating previously acquired memories). Here we demonstrate that the p38 MAPK/MAPK-activated protein kinase 2 (MK2) cascade is

required to maintain the precise tuning of long-term potentiation and long-term depression at CA1 synapses of the hippocampus which is correlated with efficient reversal learning. Using the MK2 knockout (KO) mouse, we show that mGluR-LTD, but not NMDAR-LTD, is markedly impaired in mice aged between 4-5 weeks (juvenile) to 7 months (mature adult). Although the amplitude of LTP was the same as in wildtype mice, priming of LTP by the activation of group I metabotropic receptors was impaired in MK2 KO mice. Consistent with unaltered LTP amplitude and compromised mGluR-LTD, MK2 KO mice had intact spatial learning when performing the Barnes maze task, but showed specific deficits in selecting the most efficient combination of search strategies to perform the task reversal. Findings from this study suggest that the mGluR-p38-MK2 cascade is important for cognitive flexibility by regulating LTD amplitude and the priming of LTP.

Key words

mGluR-LTD; mGluR-mediated priming of LTP; reversal learning; hippocampus; MAP kinase signalling; Barnes maze

Introduction

The steady loss of cognitive abilities is a common feature associated with both ageing and with neurodegenerative disease. These functional changes are believed to be, at least in part, due to progressive disruption of glutamatergic synaptic plasticity, which is the ability of synapses to either strengthen (long-term potentiation, LTP) or weaken (long-term depression, LTD), over a prolonged period of time (Bliss & Collingridge, 1993; Collingridge *et al.*, 2004; Sanderson *et al.*, 2016). LTP and LTD are two complementary forms of synaptic plasticity that act dynamically to mediate information storage in the brain (Neves *et al.*, 2008; Takeuchi *et al.*, 2014). In the hippocampus, two mechanistically distinct forms of LTD coexist at the Schaffer Collateral-CA1 synapse: one is induced by the activation of N-methyl-D-aspartate receptors, NMDAR-dependent LTD and the other is induced by the activation of group I metabotropic glutamate receptors, mGluR-dependent LTD (Collingridge *et al.*, 2004; Luscher & Huber, 2010;

Sanderson *et al.*, 2016). mGluR-LTD appears to be required for the acquisition of new memories and for cognitive flexibility, processes that are believed to be crucial to overwrite learned information with updated information (Xu *et al.*, 2009; Menard & Quirion, 2012; Eales *et al.*, 2014; Wall *et al.*, 2018). Recent studies using genetic modified animal models, where key molecules required for the expression of mGluR-LTD are removed (Eales *et al.*, 2014) or mutated (Mabb *et al.*, 2014; Wall *et al.*, 2018) have shown that either impairment or enhancement of mGluR-LTD is associated with deficits in the hippocampal-dependent reversal learning, with no significant deficits observed in the initial learning and acquisition phases.

The induction of either LTP or LTD requires a stimulus that surpasses a defined threshold and activates sufficient surface membrane receptors and intracellular pathways to induce the necessary structural and functional changes underlying long term plasticity. The level that the threshold is set is critical: if it is too high then no plasticity will occur disrupting learning but if it is set too low then plasticity and learning will occur in response to trivial or minor events, saturating the neural network with stored information (Takeuchi *et al.*, 2014). Thus the threshold is dynamic being modulated by previous changes in network activity. This ability of cells or synapses to register changes in activity that occurred at one point in time and use this information to adjust the threshold of subsequent synaptic plasticity, is vital for functional long-term plasticity and memory storage (Abraham, 2008). This process, termed metaplasticity, has been well-described at hippocampal glutamatergic synapses with, for example, the prior activation of mGluR regulating the induction and expression of LTP (Bortolotto *et al.*, 1994; Cohen *et al.*, 1998; Raymond *et al.*, 2000; Mellentin *et al.*, 2007; Abraham & Williams, 2008; Bortolotto *et al.*, 2008). However, it is currently unclear whether the disruption of mGluR-mediated pathways impacts on LTP itself or on the priming of the occurrence of LTP (metaplasticity) *in vivo*.

Here we show that the MAPK-activated protein kinase 2 (MK2) cascade is required for both hippocampal mGluR-LTD and the mGluR-mediated priming of LTP. MK2 is a serine/threonine kinase, highly expressed in the brain, that binds and is activated by p38 α / β isoforms (Correa & Eales, 2012; Eales *et al.*, 2014). Although the requirement of the MK2 cascade regulating inflammatory processes is well-established (Menon &

Gaestel, 2018), not much information is available about its physiological function in the brain. Using a MK2 knockout (KO) mouse line we have shown that mGluR-dependent LTD is impaired at CA1 hippocampal synapses and that these mice are unable to efficiently perform the reversal of a previously learned hippocampal-dependent task. Interestingly, MK2 KO mice have no overt deficits in either basal synaptic transmission or NMDA receptor mediated synaptic plasticity (LTP and LTD) but the mGluR-mediated priming of LTP is significantly disrupted. These findings identify the MK2 cascade as a critical pathway regulating synaptic plasticity, whose disruption leads to distinct deficits in cognitive flexibility.

Materials and Methods

MK2 knockout (KO) mice colony: Animals were kept in standard housing with littermates, provided with food and water *ad libitum* and maintained on a 12:12 (light-dark) cycle. The Barnes Maze test and the hippocampal slice experiments were performed at the University of Bradford. The animals were treated in accordance with the Animal Welfare and Ethics Review Body Committee (AWERB) and experiments were performed under the appropriated project licenses with local and national ethical approval. Samples sizes for behavioral and slice experiments were calculated using variance from previous experiments to indicate power, which statistical analysis significance was set at 95%. MK2 heterozygous mice were used for breeding to generated MK2 WT and MK2^{-/-} (KO) mice strain. The MK2^{-/-} mouse was generated as described in (Kotlyarov *et al.*, 1999). The MK2 KO mice are viable, fertile and no obvious behavioural or gross morphological abnormalities were observed.

Hippocampal slice preparation

Hippocampal slices (400 µm) were obtained from 1-to-7 month old WT and MK2 KO mice. Animals were sacrificed by cervical dislocation and decapitated in accordance with the U.K. Animals (Scientific Procedures) Act (1986). The brain was rapidly removed and placed in ice-cold high Mg²⁺, low Ca²⁺ artificial CSF (aCSF), consisting of the following (in mM): 127 NaCl, 1.9 KCl, 8 MgCl₂, 0.5 CaCl₂, 1.2 KH₂PO₄, 26 NaHCO₃, 10 D-glucose (pH 7.4 when bubbled with 95% O₂ and 5% CO₂, 300 mOSM). Parasagittal brain slices were then prepared using a Microm HM 650V microslicer in

ice-cold aCSF (2-4°C). Slices were trimmed and for the mGluR-LTD experiments the CA3 region was removed. Slices were allowed to recover at 34°C for 2-6 hrs in aCSF (1 mM MgCl₂, 2 mM CaCl₂) bubbled with 95% O₂ and 5% CO₂ before use. For mGluR-LTD experiments, hippocampal slices were allowed to recover for at least 3 hrs before use.

Electrophysiology

Extracellular recording from hippocampal slices

Field excitatory postsynaptic potentials (fEPSPs) were recorded from interleaved slices from WT and MK2 KO mice. An individual slice was transferred to the recording chamber, submerged in aCSF (composition as above), maintained at 32°C (npi-TC 20 Temperature Controller System Scientifica), and perfused at a rate of 5 ml/min. The slice was placed on a grid allowing perfusion above and below the tissue and all tubing was gas tight (to prevent loss of oxygen). To record fEPSPs, an aCSF filled microelectrode was placed on the surface of stratum radiatum in the CA1 region. A bipolar concentric stimulating electrode (FHC) controlled by an isolated pulse stimulator DS2A (Digitimer Ltd) was used to evoke fEPSPs at the Schaffer collateral–commissural pathway. The mGluR-LTD recordings were made in the presence of 50 µM picrotoxin to block GABA_A receptors (Sigma) and the NMDA receptor antagonist L-689,560 (trans-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline; 5 µM; Tocris). Stimulus input/output curves for fEPSPs were generated using stimulus strength of 1-5 V (stimuli duration 0.1 ms). Paired pulse facilitation was measured over an interval range of 50 to 500 ms. Field EPSPs were evoked at 0.1 Hz with a 20 min baseline and recorded at a stimulus intensity that gave 35-40 % of the maximal response. To pharmacologically induce mGluR-LTD 100 µM of (RS)-3,5-DHPG (3,5-dihydroxyphenylglycine, Tocris) was applied for 10 min and then washed off for at least one hour as previously described (Eales *et al.*, 2014). To synaptically induce mGluR-LTD, a paired-pulse low frequency stimuli (PP-LFS) consisting of 900 pairs pulses with a 50 ms intervals delivered at 1 Hz protocol was delivered at the Schaffer collateral, and LTP was induced using two 1 second 100 Hz bursts of stimuli which were separated by a 30 sec interval. To induce NMDA receptor-dependent long term depression (NMDAR-LTD) a low frequency stimulation (LFS)

protocol was used (900 pulses at 1 Hz) with a baseline stimulation frequency of 0.03 Hz (Dudek & Bear, 1992). Recordings were rejected if the fEPSP slope exceeded the baseline value after the LFS, as transmission was judged not to be stable.

Recordings of fEPSPs were made using a Differential Amplifier DP-301/1C with signals filtered at 3 kHz and digitized online (10 kHz) with a Shielded BNC Connector Block National Instruments interface controlled by WinLTP 2.2 software (Anderson & Collingridge, 2007). Field EPSPs were analyzed using WinLTP 2.2 software and graphs prepared using Origin (Microcal), with the slope of fEPSPs measured for a 1 ms linear region following the fiber volley.

Whole cell patch-clamp recording

AMPA receptor –mediated miniature excitatory postsynaptic currents (mEPSCs) were recorded as previously described (DaSilva *et al.*, 2016). A hippocampal slice was transferred to the bath, perfused at 2-3ml/min at 32°C. Slices were visualized using IR-DIC optics with a Slice Scope (Scientifica) microscope and CCD camera (Scientifica) at a total magnification of 400X. Whole-cell patch clamp recordings were made from CA1 pyramidal neurons using patch pipettes (5-8 M Ω) made from thick walled borosilicate glass (Harvard Apparatus) filled with (mM): 135 potassium gluconate, 7 NaCl, 10 HEPES, 0.5 EGTA, 10 phosphocreatine, 2 MgATP, 0.3 NaGTP, pH 7.2, 290 mOsm. To isolate AMPA-mediated mEPSCs, aCSF contained 1 μ M TTX, 50 μ M picrotoxin to block GABA_A receptors and 5 μ M L_{689,560} to block NMDA receptors. Recordings of mEPSCs were obtained at a holding potential of -60 mV using an Axon Multiclamp 700B amplifier (Molecular Devices), filtered at 3 kHz and digitized at 20 kHz (Digidata 1440A, Molecular Devices). Data acquisition was performed using pClamp 10 (Molecular Devices). Analysis of mEPSCs was performed using MiniAnalysis software (SynaptoSoft). Events were manually analyzed and were accepted if they had amplitude > 6 pA (events below this amplitude were difficult to distinguish from baseline noise) and had a faster rise than decay. Statistical significance was measured using a one-way ANOVA with 0.05% taken as significant.

Barnes maze task

The cohort for behavioral experiments consisted of male MK2 KO mice (n = 10) and WT littermates (n = 12). Mice were 7 month old at the beginning of training and were trained daily for a period of 17 consecutive days. Animals were provided with food and water *ad libitum* and were maintained at 12:12 h light/dark cycle as before the handling and behavioral tests began. All behavioral tests and analysis were performed with the experimenter blinded to genotype.

To test whether the MK2 KO mice were able to acquire spatial reference memory as efficiently as WT littermates we used the Barnes maze task. The experimental design, including the behavioural paradigm, and the parameters analyzed were as described in (Wall *et al.*, 2018). Briefly, hippocampal dependent spatial learning was assessed using a circular Barnes maze that measured 1 meter in diameter, was situated 1 meter from the floor, and contained 20 5-cm holes that were evenly spaced (5 cm apart) around the perimeter (Ugo Basile). The maze was positioned centrally within the laboratory; with a round plastic circle positioned 30 cm from the edge of the maze. The circle was held by a robe attached to the ceiling and positioned at 25 cm above the maze height to allow the animals a clear view of the circle from the maze surface. Four distinctly different geometric figures with contrasting colors, but similar sizes were hung from the circle at fixed positions to act as spatial cues. These cues together with other objects in the testing areas such as floor lamps, camera holder desk and recording computer were all visible to the mice from any position of the maze. The position of the cues remained unchanged throughout the acquisition and the reversal phase.

A bright white light evenly shining on the maze served as an aversive stimulus. The maze had an 'exit' box positioned under one of the holes. The 'exit' hole was randomly assigned on the first day and maintained in this position for 12 days prior to the 180° shift (see below) for the remaining 5 days of training. One week before the training started the animals were handled daily by the experimenter. From the first day of handling until the end of the tests the experimenter was the only person to be in contact with the animals. Each day mice were kept in their original cage in a dimly lit environment for at least 30 minutes prior to the test. Each mouse was then transferred to the centre of the maze within a 1 litre black plexiglass beaker. The mouse was left under the beaker for 30 seconds. During this period the strong experimental light was switched on. The beaker was then removed allowing the mice to explore the maze and

the trial started. When the beaker was removed mice were free to move randomly in any direction. The task was completed when the mouse entered the exit hole. If mouse failed to complete the task during the first 10 minutes, it was guided to the “exit” box. Once in the box mouse was then allowed to stay in the hole for 1 minute to familiarise with the environment. On days 1-5, flavoured treats were added in the exit box as a reward for task completion. On days 6-17, treats were awarded in the home cage on completion to prevent cued orientation of the exit box location via olfactory stimulation. To avoid olfactory cues, the arena and the exit box were cleaned twice with 70% ethanol first and distilled water after each trial. All behavioral runs were recorded using a camera system (Bird Box Camera with 700TVL Sony EFFIO CCD) attached to a computer for offline analysis (Any-MAZE v4.96, Stoelting Co.). Total distance, duration, speed, and accuracy of task performance were measured. Error number was measured by calculating the number of incorrect holes visited before locating the correct 'exit' hole. Mapping the progression of the animals around the maze allowed determination of the search strategy. These were: random: no consistent pattern, >2 crossings of the open field; serial: a hole-by-hole progression with ≥ 3 consecutive holes visited; and spatial: moving directly to the exit hole ± 2 holes and no deviation outside of the exit quadrant (Wall *et al.*, 2018).

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software. For electrophysiological recordings post-hoc Bonferroni test combined with one-way ANOVA were used. Behavioural data were analysed using Student's t-test and repeated-measures analysis of variance (ANOVA). For all experiments significance was set at $p \leq 0.05$. Data presented in figures are means (\pm SEM).

Results

Juvenile MK2 KO mice have impaired hippocampal mGluR-LTD.

Previous findings from our laboratory have shown that MAPK-activated protein kinases 2 and 3 (MK2/3) provide a mechanistic link between mGluR-dependent activation of p38 MAPK and the endocytosis of AMPA receptors that occurs during mGluR-LTD

(Waung *et al.*, 2008; Eales *et al.*, 2014; Mabb *et al.*, 2014; Wall *et al.*, 2018). Mice with the double knockout (DKO) of both MK2 and MK3 have compromised mGluR-LTD and also exhibit specific deficits in hippocampal-dependent spatial learning (Eales *et al.*, 2014). This previous study, using hippocampal cultured neurons, provided evidence that loss of MK2, but not MK3, may be responsible for the deficits observed in mGluR-LTD (Eales *et al.*, 2014).

To investigate in detail the role of the MK2 cascade in the cognitive deficits observed in MK2/3 DKO mice at 4-to-5 weeks- old (Eales *et al.*, 2014), field excitatory postsynaptic potentials (fEPSPs) were recorded at Schaffer collateral-CA1 synapses from interleaved hippocampal slices obtained from WT and MK2 knockout (KO) littermates at the same age. MK2 KO mice did not show any alterations in basal synaptic transmission: there was no significant ($p > 0.05$) change in the input/output relationship (Figure 1A) or the degree of paired-pulse facilitation (Figure 1B). To determine the role of the MK2 cascade in the regulation of mGluR-LTD, hippocampal slices were exposed to the specific agonist for group I metabotropic receptors (RS)-3,5-dihydroxyphenylglycine (DHPG). In agreement with previous findings (Wall *et al.*, 2018), application of DHPG (100 μ M, 10 min) to WT hippocampal slices produced a sustained depression of synaptic transmission (Figure 1C and 1D). In contrast, long-term depression did not occur in slices from MK2 KO littermates (measured at 45-55 min after DHPG washout, Figure 1C and 1D). These findings confirm our previous hypothesis, based on findings from cultured hippocampal neurons (Eales *et al.*, 2014), that MK2 is required for mGluR-LTD.

NMDA receptor dependent synaptic plasticity is unaltered in MK2 KO mice

Considering that mGluRs play an important role in regulating the induction and maintenance of LTP (Bortolotto *et al.*, 1994; Cohen *et al.*, 1998; Raymond *et al.*, 2000; Mellentin *et al.*, 2007; Abraham, 2008; Bortolotto *et al.*, 2008) and that the MK2 KO mice have impaired mGluR-LTD, we hypothesised that induction and maintenance of LTP may be altered in MK2 KO mice, where the mGluR-p38-MK2 signalling pathways are disrupted (Bolshakov *et al.*, 2000; Moulton *et al.*, 2008; Gladding *et al.*, 2009; Sanderson *et al.*, 2016).

To test this hypothesis, we recorded fEPSPs at Schaffer collateral-CA1 synapses from interleaved acute hippocampal slices obtained from 8 week-old WT and MK2 KO mice. As observed in 4-to-5-weeks old mice, no differences were seen in either input/output curve or in the paired-pulse facilitation ratio (Figures 2A and 2B) and thus basal synaptic transmission in MK2 KO mice is not altered during postnatal development. To address whether MK2 deletion impacts on LTP, we used 2 trains of HFS (2 x 1 sec trains of 100 Hz with 30 sec interval) to induce LTP in WT (6 out of 6 slices; n = 6 animals) and MK2 KO mice (7 out of 7 slices; n = 7 animals) and found no significant difference in LTP amplitudes (Figure 2C).

Since LTP is dependent on NMDA receptor activation and appeared unaffected by deletion of MK2, we next tested whether NMDA receptor-dependent long-term depression (NMDAR-LTD) was altered in MK2 KO mice at 8 weeks using a LFS protocol ((Dudek & Bear, 1992), Figure 2 D). NMDAR-LTD was induced in 8 out of 12 slices in WT mice and in 5 out of 11 slices in MK2 KO mice. Neither the short term depression (5 minutes immediately following the LFS, WT 27.2 ± 4.2 %, n = 12 slices; MK2 KO 22.6 ± 3.1 %, n = 11 slices) or LTD (measured 55-60 minutes after the LFS) were significantly different between the genotypes (Figure 2D).

This observation suggests that the mGluR-p38-MK2 signaling pathway is not directly involved in the induction of LTP or NMDAR-LTD. However, activation of the mGluR-p38-MK2 cascade may play a role in setting the threshold to induce LTP. To test this hypothesis we used a well-established protocol to activate (or prime) the mGluR signaling pathway prior to the induction of LTP (Cohen, 1998; Raymond, et al., 2000; Mellentin et al., 2007). We used the following protocol with interleaved hippocampal slices obtained from WT and MK2 KO mice: in a WT slice LTP was induced under control conditions (induced with 2 trains of 100 Hz stimuli with a 30 sec interval); and the next slice was exposed to DHPG (20 μ M) for 10 min to prime intracellular mGluR-dependent signaling. After DHPG was washed out for 20 min, LTP was then induced as before (Figure 2C). The same sequence was used for slices from MK2 KO mice. The application of 20 μ M DHPG produced a small (~ 30 %) reversible reduction in fEPSP slope which was not significantly different between WT and MK2 KO slices (Figure 2E).

In agreement with previous findings, we observed a significant increase in LTP amplitude in WT slices during induction (Figure 2E; first 2-3 min) and maintenance (40-50 min after LTP induction) in slices that had been pre-treated with DHPG (20 μ M; 10 min) compared to naïve slices (Figure 2C and 2E). Strikingly, no changes in LTP amplitude were observed in slices obtained from MK2 KO mice that had been exposed to DHPG (Figures 2C and 2E). These findings showed, that in WT slices, activation of mGluR enhances LTP amplitude, effect that is disrupted in MK2 KO mice (there was a significant increase ($p = 0.0015$) in the potentiation of fEPSP slope (LTP) between 70 to 80 minutes after HFS (WT: 122.4 ± 16.1 %; MK2 KO: 41.0 ± 3.8 %; Figure 2F). These findings suggest that the mGluR-p38-MK2 cascade is required for the priming of LTP and thus provides a novel molecular mechanism for metaplasticity.

MK2 KO mice exhibit deficits in synaptically-induced mGluR-LTD and reversal learning at 7 months of age.

Previously, we have shown that MK2/3 DKO mice, which have deficits in hippocampal mGluR-LTD, retain the capacity to complete a hippocampal-dependent spatial learning task (the Barnes maze), but have poor performance during reversal learning (Eales *et al.*, 2014). These experiments were performed in juvenile (4-to 5-week old) mice. We have shown in the present study that juvenile MK2 KO mice have deficits in hippocampal mGluR-LTD ((Eales *et al.*, 2014); Figure 1C) that are very similar to findings observed in MK2/3 DKO mice confirming that MK2 is required for mGluR-LTD. Here we wanted to test whether the deficits in mGluR-LTD and metaplasticity that we observed in juvenile MK2 KO mice led to changes in basal synaptic transmission and plasticity at more mature ages. To address this, we first recorded extracellular field excitatory potentials at the Schaffer collateral-CA1 synapses from interleaved slices obtained from 7 month old WT and MK2 KO mice. Firstly, we noted that there was a small but significant reduction in the input/output curve in MK2 KO compared to WT mice (Figure 3 A(i)). However, the analysis of the fibre volley (Figure 3A(ii) and 3B), was not significantly different between the two genotypes suggesting that the difference observed in the input/output curve was not due to a differential recruitment of fibres. Similarly, the strength of synapses is comparable, as revealed in

the paired pulse ratio (Figure 3C) showing no change in the release probability between the two genotypes.

To investigate basal synaptic transmission in more detail (Figure 3A-C), we recorded AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) in CA1 pyramidal cells in interleaved slices from 7 month old mice MK2 KO and WT slices. There was no significant difference in either the amplitude of mEPSCs or the time interval between mEPSCs (Figure 3D and 3E). There was also no significant ($p = 0.95$) difference in mEPSC rise (10-90 % rise-time WT 1.77 ± 0.2 ms compared to 1.75 ± 0.2 ms) or decay kinetics (Figure 3F; $p = 0.52$, decay fitted with single exponential, WT $\tau = 8.2 \pm 0.4$ ms, MK2 KO $\tau = 8.7 \pm 0.6$ ms) between the genotypes. The lack of difference in mEPSC amplitude and frequency suggests there is no difference in either receptor number or transmitter release probability.

The mechanisms regulating hippocampal mGluR-LTD expression during the first 5 weeks of animal life have been previously described (Nosyreva & Huber, 2005). However, not much information is available on the expression of mGluR-LTD in mature adults (animals older than 2 months). To address this we applied PP-LFS at the Schaffer collaterals (900 paired-pulses delivered at 1 Hz, with an inter-pulse interval of 50 ms) to induce mGluR-LTD in the CA1 region of the hippocampus of WT and MK2 KO mice at 7 months of age. Using this protocol we observed that synaptically-induced mGluR-LTD is also impaired in slices from MK2 KO mice compared to slices from WT mice (Figure 3G and 3H). These findings show that the MK2 cascade is implicated in both mGluR-mediated synaptic plasticity and metaplasticity processes in the hippocampus which persist from juvenile to adult.

Previously, we have shown that MK2/3 DKO mice, which have impaired mGluR-LTD, are still able to learn a hippocampal-dependent spatial task (Barnes maze; (Bach *et al.*, 1995)), but displayed clear deficits during task reversal (Eales *et al.*, 2014). Recently, we have shown that another mutant mouse that had enhanced mGluR-LTD is also able to learn the Barnes maze task, but displayed deficits in reversal learning (Wall *et al.*, 2018). In particular, these mice showed specific impairments in using the most efficient search strategy to perform the task reversal, consistent with a lack of cognitive

flexibility (Wall *et al.*, 2018). These findings suggest that expression of the correct level of mGluR-LTD is associated with successful reversal learning and with an ability to select the most efficient search strategy.

From these previous findings, we predicted that the reversal of a spatial learned task will be impaired in MK2 KO mice, but it is unclear whether the deficits will resemble those observed in mice in which mGluR-LTD is enhanced (Wall *et al.*, 2018). To investigate this we used the Barnes maze paradigm to test the ability of WT and MK2 KO mice to learn a spatial reference task (Eales *et al.*, 2014; Wall *et al.*, 2018). Mice were tested for 17 consecutive days, grouped into acquisition, consolidation, and expression phases of learning (days 1 to 12) and reversal acquisition of the previous learned phase (days 13 to 17). During spatial acquisition (days 1 to 12) no differences were observed between the genotypes for the time spent to find the 'exit' hole or the number of errors made to find the exit location (Figure 4A and 4B; learning task). On day 13, the location of the exit box was moved to the opposite side of the maze (exit hole location was rotated of 180°), requiring the mice to learn the new location for the exit hole (reversal learning). Although WT and MK2 KO mice performed similarly in the learning and acquisition phases, the MK2 KO mice showed a significant impairment to learn the new location of the exit during the reversal phase. The MK2 KO mice spent a longer time to find the exit hole (Figure 4A; reversal) and also made a significantly larger number of errors to learn the new 'exit' hole location (Figure 4B, reversal). Thus, the MK2 KO mice are able to learn the spatial reference task as well as the WT mice, but show impairments in performing the reversal task.

We next examined the strategy used to search for the maze exit hole during both learning and reversal. Both WT and MK2 KO mice showed a similar ability to shift from a combination of random and serial strategies to a spatial search strategy during the learning process (days 1-12; Figure 4). However, when the exit hole was rotated 180° (reversal learning), there were clear differences in the search strategy employed by WT and MK2 KO mice. On the day of reversal (day 13), WT and MK2 KO mice used similar search strategies to the first day of training (day 1; Figures 4C and 4E). As the animals were tested 24 hours after 12 consecutive days of training, the first day of

reversal (day 13) can be used as a probe to assess long-term memory acquisition. No difference in performance was observed between genotypes on day 13 (day 13; Figure 4A and 4C and 4E), suggesting that they are equally able to learn and memorize the spatial task. However, in subsequent days, WT mice replaced random and serial for a spatial search strategy (as observed during learning before task reversal). In contrast, MK2 KO mice were not able to reduce the proportion of time spent carrying out the random strategy during reversal (Figure 4C-E) and showed a significant delay in employing the spatial search strategy throughout the reversal phase (Figure 4C and 4D; days 14 to 17). Taken together, this data shows that MK2 KO mice are unable to reuse strategic approaches previously acquired during task learning, consistent with cognitive inflexibility.

Discussion

In this study we have for the first time characterised synaptic transmission, synaptic plasticity and learning behaviour in mice in which the MAPK-activated protein kinase 2 (MK2) has been deleted. Although the MK2 deletion was global and constitutive, there were no obvious locomotor or behavioural abnormalities, mice were fertile and did not have a reduced life span. We specifically investigated changes in basal synaptic transmission, synaptic plasticity and metaplasticity in the hippocampus and then have assessed the effect that MK2 deletion has on learning a spatial task that is hippocampal dependent. There were no overt differences in basal synaptic transmission in the hippocampus of juvenile mice (4-5 weeks) or in adult mice (7 months of age). Deletion of MK2 had no significant effect on NMDAR-dependent synaptic plasticity: the induction and amplitude of LTP and NMDAR-dependent LTD were not altered compared to WT littermates.

In contrast to LTP, where the link to specific learning and memory tasks is well established (Neves *et al.*, 2008; Takeuchi *et al.*, 2014), the importance and role of mGluR-LTD in learning and memory processes is only now beginning to emerge (Sanderson *et al.*, 2016). Recent evidence has linked alterations in mGluR-dependent LTD with specific deficits in performing the reversal of a previously learned task (Eales

et al., 2014; Mills *et al.*, 2014; Wall *et al.*, 2018). Therefore, mapping the molecular mechanisms underpinning mGluR-LTD is critical to understand the mechanism by which previously learned information can be updated or replaced. Here we provide evidence that the MK2 cascade is critical for the induction of mGluR-LTD (either chemically induced with DHPG or synaptically induced with a paired pulse LFS protocol), an effect that persists across postnatal development. The MK2 cascade is also required for the mGluR-mediated priming of LTP and disruption of the MK2 cascade results in specific alterations in the processes required for efficient task reversal.

The importance of the MK2 and MK3 cascades regulating synaptic plasticity and learning and memory is still not well characterized. Recent findings showed that knockout of both MK2 and MK3 kinases resulted in impaired hippocampal mGluR-LTD and that MK2/3 double KO mice retained the capacity to acquire a hippocampal-dependent spatial task (Barnes maze) but were unable to learn the task reversal (Eales *et al.*, 2014). However, as this study used a double knockout of MK2 and MK3, the specific requirement for either MK2 or MK3 in synaptic plasticity remained uncharacterized. The present study addresses this issue and shows that loss of MK2 alone results in clear deficits in mGluR-LTD. Neither DHPG or paired-pulse low frequency stimuli could induce LTD at CA1 synapses of the hippocampus of juvenile (4-5 week-old) or 7 month old MK2 KO mice. Furthermore, the deletion of MK2 mediates mGluR priming of LTP as well as the magnitude of LTP. It is however not possible to discount some role for MK3 in these processes without utilising an MK3 KO mouse.

Recent reports have shown that alterations in mGluR-LTD correlate with impairments in task reversal (Eales *et al.*, 2014; Mills *et al.*, 2014; Wall *et al.*, 2018). MK2 KO mice, which have intact LTP but impaired mGluR-LTD, are still able to learn the Barnes maze task, being indistinguishable from WT mice in the acquisition, consolidation and expression phases. However, MK2 KO mice are unable to select the most efficient search strategies to perform the reversal of the task when compared to WT littermates. During reversal learning WT mice steadily replace the random searching strategy with the spatial strategy (Figure 4C and 4D). The increase in the use of the spatial search

strategy presumably occurs as the mice replace memories of the spatial cues associated with the original position of the 'exit' hole with memories of the new relationship between spatial cues associated with the re-positioned 'exit' hole. In contrast, the MK2 KO mice persist with the random search strategy, which leads to a marked increase in the number of errors (Figures 4B, 4D and 4E). Thus, it appears that the MK2 KO mice cannot efficiently assimilate the new relationship between spatial cues and the re-positioned 'exit' hole.

MK2 KO mice, which have impaired mGluR-LTD and impaired mGluR-mediated priming of LTP, showed marked deficits in selecting the most efficient search strategy to perform the reversal of a previously learned spatial task. Surprisingly, ArcKR mice, which have enhanced mGluR-LTD, also display deficits in selecting search strategies during the Barnes maze reversal task (Wall *et al.*, 2018). These findings are consistent with hypothesis that the correct amount of mGluR-LTD is required for efficient task reversal with either too much or too little leading to learning impairments. Interestingly, the specific deficits in task reversal appear different between mice with impaired mGluR-LTD (MK2 KO) and mice with enhanced mGluR-LTD (ArcKR). The ArcKR mice were not able to employ random and spatial search strategies and instead used only a serial strategy to find the 'exit' hole (Wall *et al.*, 2018). In contrast (as outlined above) the MK2 KO mice overuse the random search strategy. One plausible explanation for these differences is that the amplitude of mGluR-LTD plays a key role in determining the selection of search strategies during task reversal. An alternative explanation for the difference in observed learning behaviours in MK2 KO compared to ArcKR mice could be the lack of mGluR-dependent priming of LTP in MK2 KO mice. Currently it is unknown if induction of LTP is altered in the ArcKR mice. Furthermore, the status of the mGluR-mediated priming of LTP in ArcKR mice is unclear but it is unlikely to be abolished, as the mGluR-mediated signalling pathways are intact.

Although changes in mGluR-LTD correlate with impairments in reversal learning, the precise neural correlate of reversal learning has not been defined and is probably complex consisting of multiple processes. To date there is no compelling data that outline the precise roles that the different forms of synaptic plasticity LTP and LTD

(mGluR and NMDAR-dependent) play during learning and task reversal and whether metaplasticity (mGluR-mediated priming of LTP) is required for the ability to switch tasks. It is reasonable to speculate that LTD is involved in modifying existing memories and LTP is required for the acquisition of new memories (Neves *et al.*, 2008; Takeuchi *et al.*, 2014). However there is no definitive evidence to support this speculation. The uncertainty stems from an inability to selectively distinguish between the different forms of plasticity/metaplasticity in *in vitro* and in behaving animals. For example inhibition of NMDA receptors will impair both LTP and NMDAR-mediated-LTD. However, the molecular mechanisms and signalling pathways underlying NMDAR-mediated LTP and LTD are distinct. For example, it has been shown that the janus kinase /signal transducer and activator of transcription (JAK/STAT) / inhibitor AG490 blocks NMDAR-mediated LTD induced by low frequency stimulation but has no effect on LTP (Nicolas *et al.*, 2012). Therefore this inhibitor could be a powerful tool to investigate the role of NMDAR-LTD and LTP in behaving animals, although it has currently not been used in such experiments. Another possible approach is to use specific inducible gene knockout animals. However, it is difficult to target genes that are only involved in one form of synaptic plasticity. For example previous findings showed that mice lacking mGlu5 displayed significant deficits in task reversal when performing the Morris water maze task (Xu *et al.*, 2009). Although this suggests a role for mGluR-LTD in reversal learning it is likely that mGluR-dependent priming of LTP is also affected. Thus again, it is not possible to be certain which specific form of plasticity are important for reversal learning.

Findings from this study have shown that disrupting the mGluR-p38-MK2 pathway is sufficient to block both mGluR-LTD and the mGluR-mediated priming of LTP, but the NMDAR-mediated synaptic plasticity appears unaltered. These findings are of particular interest as the p38-MK2 pathway is also critically involved in the production of pro-inflammatory cytokines and are activated in immune cells of the brain during inflammation (Heneka *et al.*, 2014). Pro-inflammatory factors, such as tumor necrosis factor alpha (TNF α), have been shown to regulate glutamatergic synaptic plasticity and cognition (Beattie *et al.*, 2002; Stellwagen & Malenka, 2006; Hogg *et al.*, 2016; Santello *et al.*, 2019). These observations place the p38-MK2 cascade as a potential

mechanistic link between inflammatory processes and synaptic dysfunction observed in ageing and neurodegenerative diseases. As the molecular components of the p38-MK2 cascade are ubiquitous expressed in the brain more investigation is required to dissect whether impairments in synaptic function result by activating the MK2 cascade in neurons and/or brain immune cells. Dissecting the mechanism by which the MK2 cascade provides a link between inflammatory processes and synaptic dysfunction place the MK2 pathway a potential therapeutic target to treat neuro-inflammatory diseases.

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Author Contributions

S.A.L.C. designed the project, obtained the funding and wrote the manuscript. L.P. performed and analysed the extracellular electrophysiological recordings and the behavioural testing; E.L.H. organized the animal colony including breeding and genotyping, performed extracellular electrophysiological recordings and analysed the data; G. D. helped with some NMDAR-LTD experiments and M.J.W. performed and analysed the patch-clamp recordings and NMDAR-LTD experiments and revised the manuscript, M.G. provided the MK2 knockout mice.

Declaration of interest

The author(s) confirm that this article content has no conflict of interest.

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Figure legends

Figure 1. Juvenile MK2 KO mice have impaired hippocampal mGluR-LTD

(A) Mean fEPSP slope plotted against stimulus strength for WT (n = 6 animals, 12 slices) and MK2KO (n= 6 animals, 12 slices) littermates with inset, examples of superimposed average fEPSPs traces at different stimulus strength (1 to 5 V). No significant difference was observed across the two genotypes (at any stimulus strength).

(B) Mean paired pulse ratio plotted against paired pulse interval for WT (n= 6 animals, 12 slices), and MK2KO (n= 6 animals, 12 slices) mice. No significant difference was observed across the genotypes (at any of the intervals). *Inset*, representative traces at an interval of 50 ms from WT and MK2 KO littermates.

(C) Normalised mean fEPSP slope plotted against time for WT and MK2 KO mice. After a 20 minute stable baseline (fEPSPs were stimulated at 40 % maximum), DHPG (100 μ M) was applied for 10 minutes and then washed out for 1 hour. The slope of fEPSPs was normalized to the baseline. The DHPG induced-LTD was analysed between 45-55 minutes after DHPG application. LTD (relative to the preceding baseline) was significantly impaired in

slices from MK2KO mice ($n = 6$ animals, 6 slices; $94.6 \pm 1.5 \%$) when compared to WT littermates ($n = 6$ animals, 7 slices; $67.9 \pm 2.1 \%$), $p = 0.0000093$. Representative fEPSPs traces taken at the times indicated by the numerals in the plot below (1, 10 min and 2, 50 min). Values represent mean \pm S.E.M. (D) mean percentage reduction in fEPSP slope (LTD) measured between 45 to 55 minutes after DHPG application for WT (reduction $32.1 \pm 2.4 \%$) and MK2 KO (reduction of $5.4 \pm 1.3 \%$) $p = 0.00000054$. Data are represented as mean \pm SEM. Statistical comparisons were performed with one-way ANOVA and repeated measures ANOVA for input/output curve and paired pulse facilitation ratio (genotype effect). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$

Figure 2. Characterisation of NMDAR mediated synaptic plasticity in the hippocampus of MK2 KO mice.

(A) Mean fEPSP slope plotted against stimulus strength for WT ($n = 7$ animals, 13 slices) and MK2 KO ($n = 6$ animals, 14 slices). *Inset*, superimposed averaged fEPSPs at different stimulus strengths (1 to 5 V) from WT and MK2KO littermates. There was no significant difference between genotypes (at any stimulus strength). (B) Mean paired pulse ratio plotted against paired pulse interval for WT ($n = 7$ animals, 12 slices), and MK2 KO ($n = 6$ animals, 11 slices). No significant difference was observed between genotypes (at any interval). *Inset*, representative average fEPSP waveforms (paired pulse interval of 50 ms) from WT and MK2 KO mice. (C) Normalised mean fEPSP slope plotted against time for WT and MK2 KO mice with representative waveforms taken at the times indicated by the numerals in the plot below. After a 20 minutes baseline (fEPSPs were stimulated at 40 % maximum), high frequency stimulation (HFS) consisting of 2 X 1s (100 Hz) stimulations 30 sec apart, was used to induce LTP and fEPSPs were recorded for 1 hour after HFS. The slope of fEPSPs was normalized to the baseline, and potentiation was analysed at 40-50 minutes after stimulation. No significant difference $p = 0.99$ in the amplitude of LTP was observed across the two genotypes (WT: $n = 6$ animals, 6 slices; $141.6 \pm 27.5 \%$; MK2 KO: $n = 6$ animals, 7 slices; $141.8 \pm 20.0 \%$). (D) NMDA receptor dependent-LTD was induced in slices from WT and MK2 KO mice with low frequency stimulation (LFS, 900 pulses at 1 Hz). Normalised mean fEPSP slope plotted against time for WT and MK2 KO mice. After a 20 minutes of baseline (fEPSPs were stimulated at 40 % maximum at 0.03 Hz), LTD

was induced with the LFS and fEPSPs were recorded for 1 hour after LFS. The slope of fEPSPs was normalized to the baseline, and depression was analysed at 55-60 minutes after the LFS. Representative average fEPSP waveforms are taken at the times indicated by the numerals in the plot below. NMDAR-LTD was induced in 8 out of 12 slices from WT mice and 5 out of 11 slices from MK2 KO mice. *Inset*, bar graph showing that there is no significant difference ($p = 0.687$) in the mean percentage LTD in WT mice ($23.4 \pm 6.4 \%$, $n = 8$ slices, 4 animals) and in MK2 KO mice ($20.3 \pm 6.3 \%$, $n = 5$ slices, 3 animals). (E) To assess mGluR-dependent priming of LTP, low concentrations of DHPG were applied before the induction of LTP. Normalised mean fEPSP slope plotted against time for WT and MK2 KO mice, with representative fEPSP waveforms taken at the times indicated by the numerals in the plot below. Following a 20 minutes baseline (fEPSPs were stimulated at 40 % maximum), DHPG (20 μ M) was applied for 10 minutes followed by 20 minutes washout and HFS was used to induce LTP. The slope of fEPSPs was normalized to the baseline, and the amplitude of LTP was analysed at 40-50 minutes after high frequency stimulation (70-80 minutes after DHPG application). Application of DHPG (20 μ M) caused inhibition of fEPSPs but this reduction was not different between genotypes $p = 0.2543$, (% of baseline slope: WT: $88.3 \pm 7.8 \%$; MK2 KO: $77.1 \pm 5.3 \%$). (F) Bar graph showing the significant ($p = 0.000322$) reduction in mGluR-dependent priming of LTP for MK2 KO mice vs WT mice (WT: $n = 6$ animals, 7 slices; $122.9 \pm 16.1 \%$ and MK2KO: $n = 6$ animals, 7 slices; $40.9 \pm 3.8 \%$). Data are represented as mean \pm SEM. Statistical comparisons were performed with one-way ANOVA and repeated measures ANOVA for input/output curve and paired pulse facilitation ratio (genotype effect). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Figure 3. MK2 KO mice exhibit deficits in synaptically-induced mGluR-LTD at 7 months of age.

(Ai) Mean fEPSP slope plotted against stimulus strength for WT ($n = 12$ animals, 19 slices) and MK2 KO ($n = 8$ animals, 13 slices). A significant reduction in the fEPSP slope was seen in MK2 KO compared to WT mice ($p = 0.01251$). (Aii) Mean fibre volley plotted against stimulus strength for WT and MK2 mice. (B) Mean fEPSP slope plotted against mean fibre volley amplitude for WT and MK2 KO mice. There was no

significant difference between the genotypes. *Inset*, examples of superimposed fEPSP waveforms at different stimulus strength (1 to 5 V) from WT and MK2 KO mice. (C) The mean paired pulse ratio plotted against paired pulse interval for WT (n = 12 animals, 15 slices) and MK2 KO (n = 8 animals, 13 slices). No significant difference was observed between the genotypes. *Inset*, averaged fEPSPs traces with a paired pulse interval of 50 ms from WT and MK2 KO mice. (D, top panel) Examples of averaged AMPA receptor-mediated miniature excitatory postsynaptic current (mEPSC) waveforms recorded from a WT and MK2 KO slice. Waveforms are the average of at least 50 mEPSCs superimposed on the mid-point of the rising phase. (D, bottom panel) Summary of mEPSC amplitudes and inter-event intervals from WT and MK2 KO slices. There was no significant difference in the mean mEPSC amplitude (mean mEPSC amplitude, WT 10.3 ± 0.2 mV, n = 14; MK2 KO, 10.2 ± 0.4 mV n = 11) or mean mEPSC interval (mean mEPSC interval, WT 5.9 ± 0.6 ms, n = 14; MK2 KO, 6.2 ± 1.4 ms n = 11) between the genotypes (E) Cumulative probability distributions for mEPSC amplitude and mEPSC intervals. (F) Average mEPSC waveforms recorded from WT and MK2 KO slices. The mEPSC decay is fitted with a single exponential with a time constant (τ) of 8.2 ms for WT and 7.9 ms for MK2 KO. (G) Normalised mean fEPSP slope plotted against time for WT (n = 7 animals; 8 slices) and MK2 KO (n = 7 animals, 8 slices) mice. Following a 20 minute baseline (fEPSPs were stimulated at 40% of the maximum), PP-LFS (900 paired pulses delivered at 1 Hz, with 50 ms inter-pulse interval) was used to induce LTD with fEPSPs recorded for at least 1 hr after stimulation. Representative average fEPSP waveforms are taken between 10-15 min (I) and LTD at 45-55 minutes (H) Bar graph showing the significant reduction (p = 0.0016) in the fEPSP slope (LTD) between 45 to 55 minutes after PP-LFS (MK2 KO: $5.0 \pm 1.2\%$ and WT: $19.9 \pm 3.6\%$). Data are represented as mean \pm SEM. Statistical comparisons were performed with one-way analysis of variance (ANOVA) and repeated measures ANOVA for input/output curve and paired pulse facilitation ratio (genotype effect). *p<0.05; **p<0.01; ***p<0.005.

Figure 4. Cognitive flexibility is impaired in 7 months old MK2 KO mice

(A) The average time that mice required to complete the task plotted against the day number (WT: n = 12 and MK2 KO: n = 10). No significant differences were observed

during the learning process, but there is a significant difference during the reversal phase (WT=1.2 ± 0.40 min; MK2 KO 3.6 ± 0.4 min; $p = 0.0065$). (B) The mean number of errors (incorrect holes visited) for WT and MK2 KO mice that occurred during days 1-12 (learning phase) and during days 13-17 (reversal phase). MK2 KO mice made a significantly higher number of errors during the reversal phase compared to WT (WT = 5.3 ± 2.9 , MK2 KO = 9.8 ± 2.7; $t(8) = 2.39$, $p = 0.044$). (C) Graph summarising the percentage of the time that WT (left) and MK2 KO mice (right) used random (red), serial (blue), and spatial (black) search strategies ($n = 6$ mice for both genotypes). (D) The mean frequency that the strategies from (C) were employed. During reversal, WT mice spent significantly more time using the spatial strategy compared to MK2 KO mice (WT = 52.7 ± 8.1 %; MK2 KO 21.1±8.1 %; $p = 0.033$). In contrast, MK2 KO mice used the random strategy at a significantly higher frequency compared to WT mice (WT = 20.7 ± 3.8%, MK2KO 43.9 ± 3.7%; $p = .0047$). (E) MK2 KO mice predominantly select random strategy to perform the task reversal. Maps plotting hole visitation of a representative WT and MK2 KO mouse on day 1 (novelty), day 12 (task embedded), day 13 (180° rotation) and day 17 (final day). Statistical comparisons for the duration and strategy (genotype effect) were performed using repeated measures ANOVA and independent t-tests were used to compare the number of errors. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

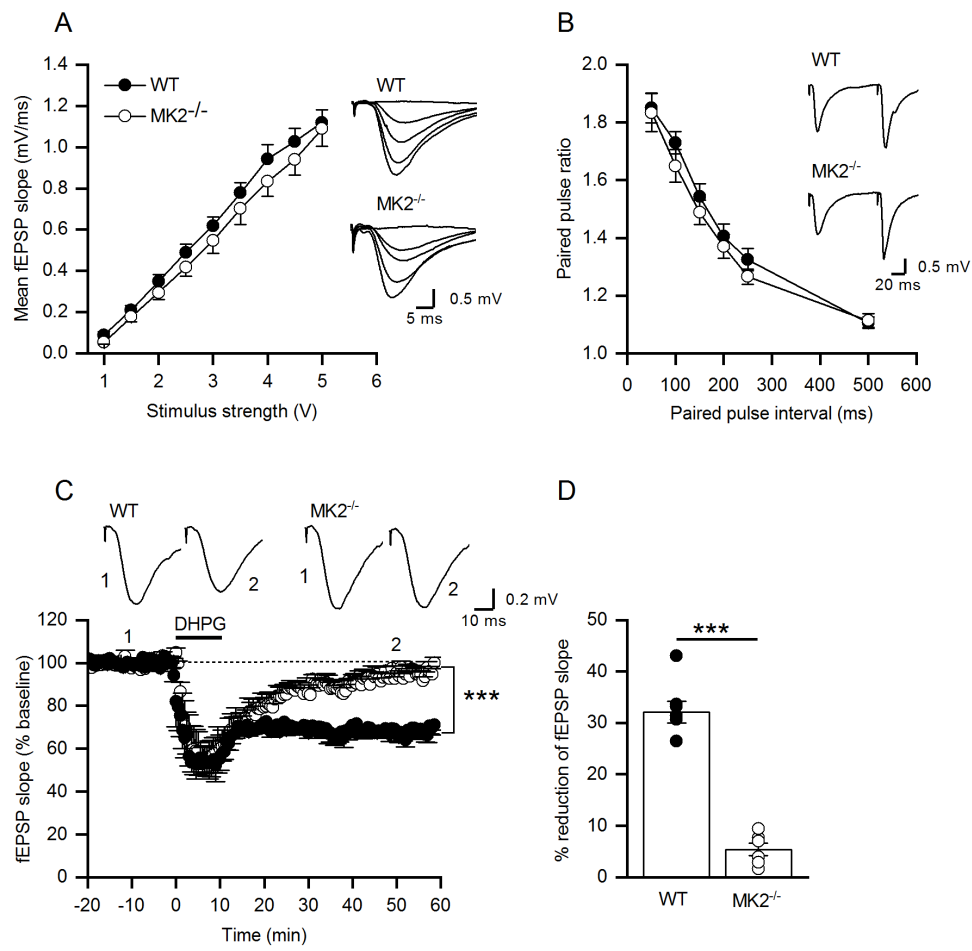


Figure 1

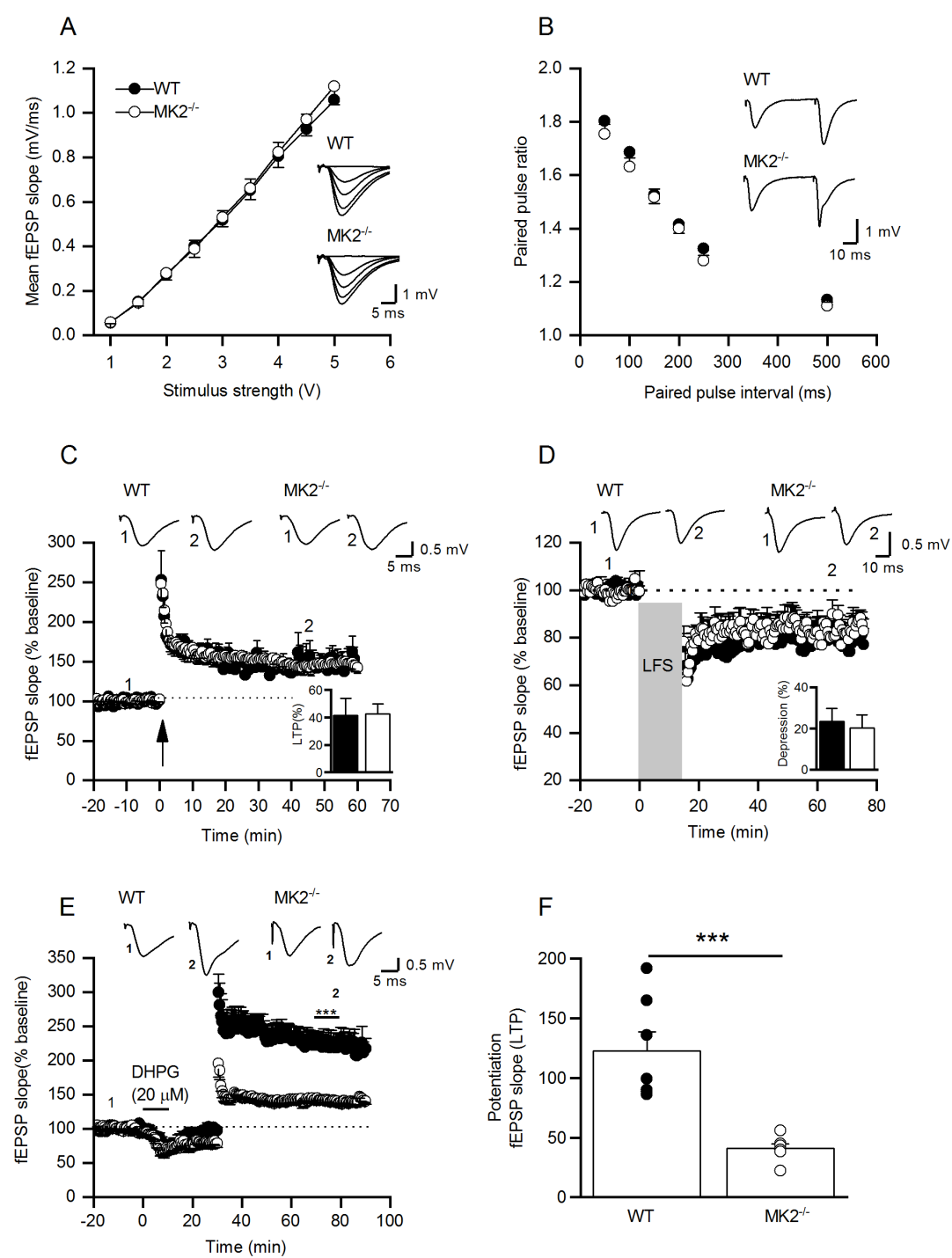


Figure 2

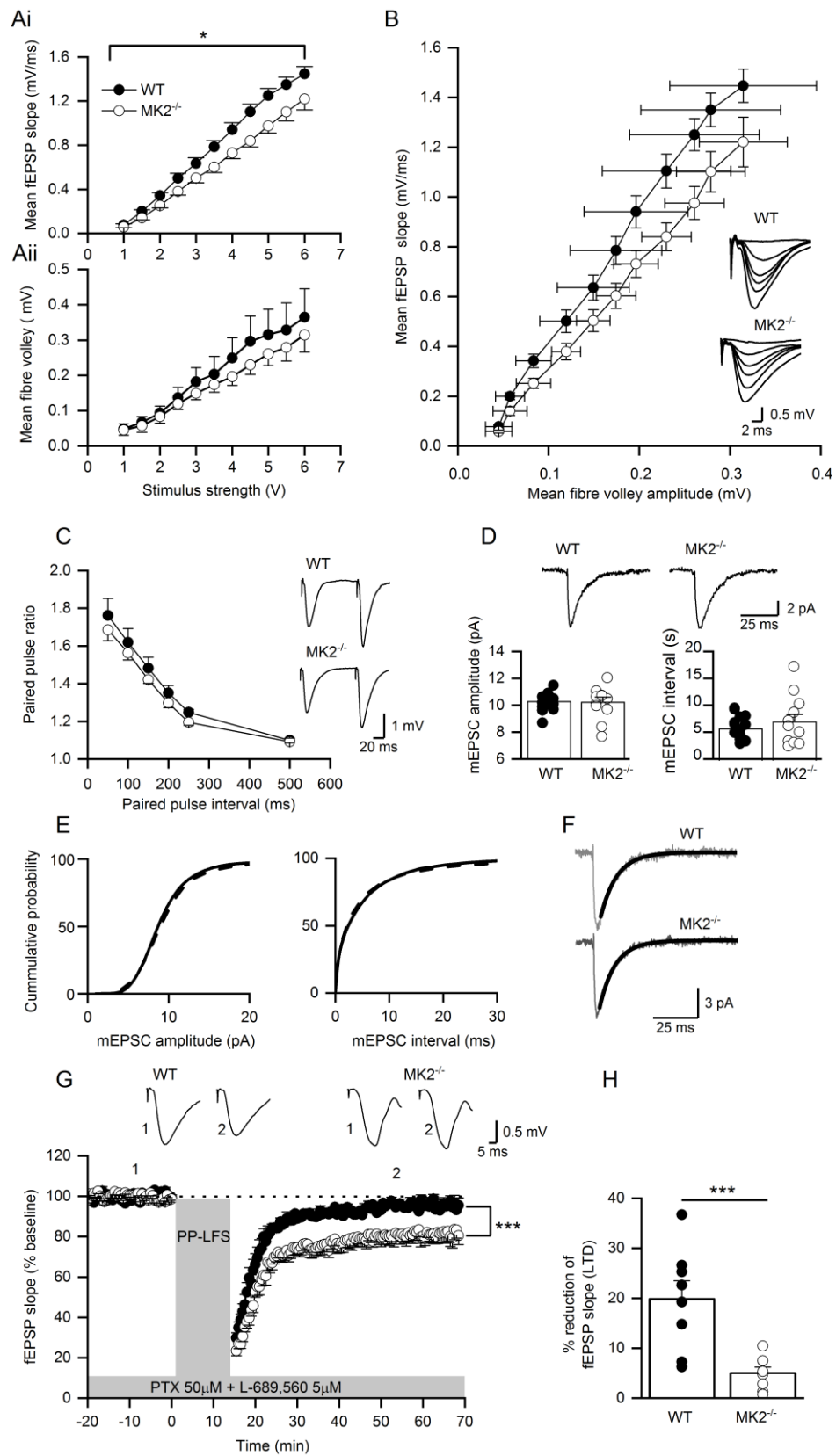


Figure 3

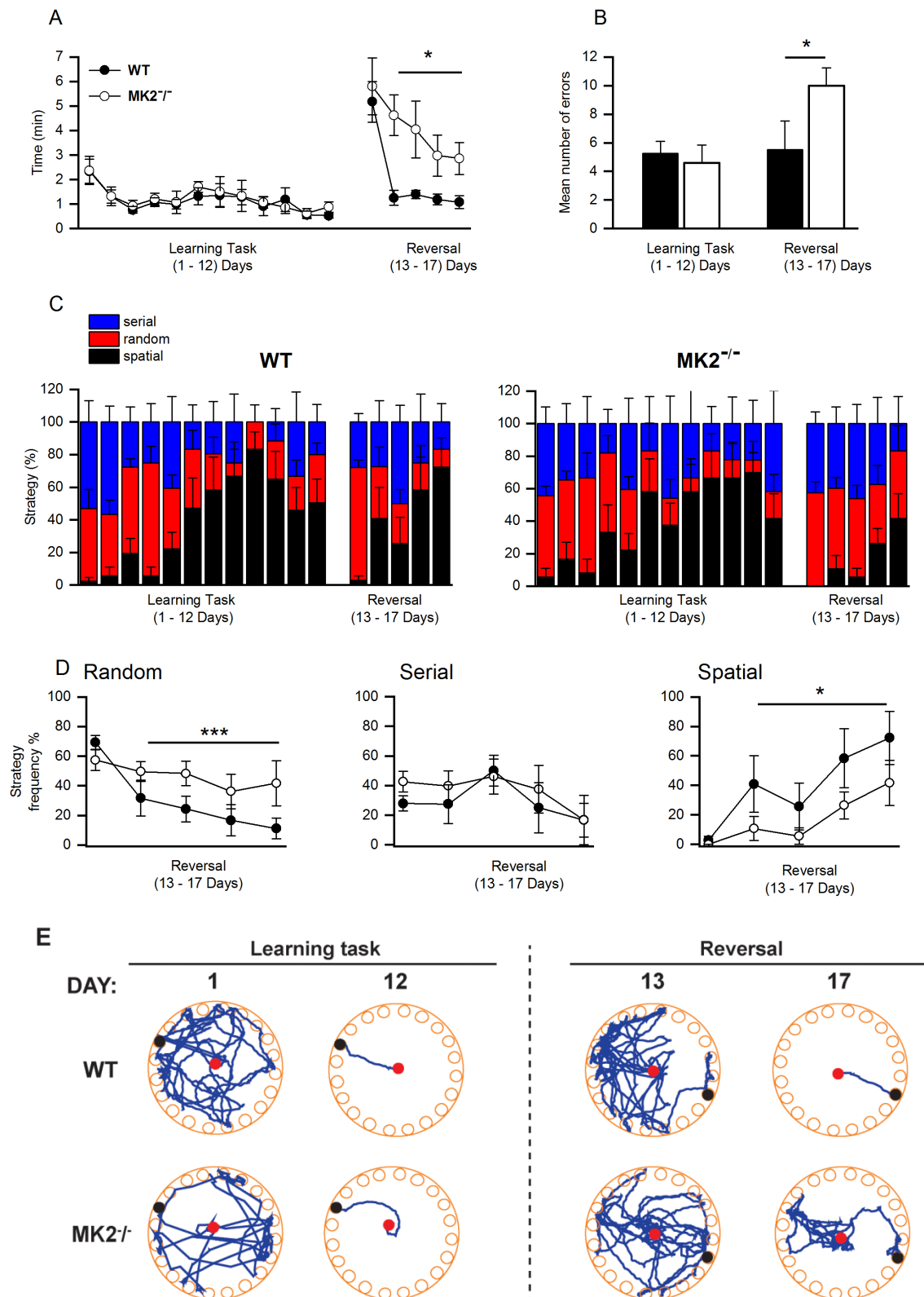


Figure 4